

	CHO	Chinese Hamster Ovary cells
	CV	Crossflow Velocity
	DFF	Direct Flow Filtration
	DV	Diafiltration Volume
5	IEF	Isoelectric Focusing
	GMH	Mass Flux (grams/m ² /hour) – also J _M
	LMH	Liquid Flux (liters/m ² /hour) – also J _L
	LPM	Liters Per Minute
	M	Molar
10	MF	Microfiltration
	NMWCO	Nominal Molecular Weight Cut Off
	NWP	Normalized Water Permeability
	PES	Poly(ether)-sulfone
15	pH	A term used to describe the hydrogen-ion activity of a chemical or compound according to well-known scientific parameters.
	PPM	Parts Per Million
	SDS-PAGE	SDS (sodium dodecyl sulfate) Poly-Acrylamide Gel electrophoresis
20	SEC	Size Exclusion Chromatography
	TFF	Tangential Flow Filtration
	PEG	Polyethylene glycol
	TMP	Transmembrane Pressure
	UF	Ultrafiltration
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Explanation of Terms:

Clarification

- 30 The removal of particulate matter from a solution so that the solution is able to pass through a 0.2 µm membrane.

Colloids

- 35 Refers to large molecules that do not pass readily across capillary walls. These compounds exert an oncotic (i.e., they attract fluid) load and are usually administered to restore intravascular volume and improve tissue perfusion.

Concentration

- 40 The removal of water and small molecules with a membrane such that the ratio of retained molecules to small molecules increases.

Concentration Polarization

- 45 The accumulation of the retained molecules (gel layer) on the surface of the membrane caused by a combination of factors: transmembrane pressure, crossflow velocity, sample viscosity, and solute concentration.

Crossflow Velocity

- Velocity of the fluid across the top of the membrane surface. CF = Pi - Po where Pi is pressure at the inlet and Po is pressure at the outlet and is related to the retentate flow rate.

Diafiltration

The fractionation process of washing smaller molecules through a membrane, leaving the larger molecule of interest in the retentate. It is a convenient and efficient technique for removing or exchanging salts, removing detergents, separating free from bound

- 5 molecules, removing low molecular weight materials, or rapidly changing the ionic or pH environment. The process typically employs a microfiltration membrane that is employed to remove a product of interest from a slurry while maintaining the slurry concentration as a constant.

10 **Feedstream**

The raw material or raw solution provided for a process or method and containing a protein of interest and which may also contain various contaminants including microorganisms, viruses and cell fragments.

15 **Filtrate Flux (J)**

The rate at which a portion of the sample has passed through the membrane.

Flow Velocity (V)

- 20 The speed at which the fluid passes the surface of the membrane is considered the fluid flow velocity. Product flux will be measured as flow velocity is varied. The relationship between the two variables will allow us to determine an optimal operational window for the flow.

Fractionation

- 25 The preferential separation of molecules based on a physical or chemical moiety.

Gel Layer

- 30 The microscopically thin layer of molecules that can form on the top of a membrane. It can affect retention of molecules by clogging the membrane surface and thereby reduce the filtrate flow.

Nominal Molecular Weight Cut Off (NMWCO)

- 35 The size (kilodaltons) designation for the ultrafiltration membranes. The MWCO is defined as the molecular weight of the globular protein that is 90% retained by the membrane.

Normalized Water Permeability (NWP)

- 40 The water filtrate flow rate established at a specific recirculation rate during TFF device initial cleaning. This value is used to calculate membrane recovery.

45 **Molecule of Interest**

- 45 Particles or other species of molecule that are to be separated from a solution or suspension in a fluid, e.g., a liquid. The particles or molecules of interest are separated from the fluid and, in most instances, from other particles or molecules in the fluid. The size of the molecule of interest to be separated will determine the pore size of the membrane to be utilized. Preferably, the molecules of interest are of biological or biochemical origin or produced by transgenic or *in vitro* processes and include proteins, peptides, polypeptides, antibodies or antibody fragments. Examples of preferred feedstream origins include mammalian milk, mammalian cell culture and microorganism cell culture such as bacteria, fungi, and yeast. It should also be noted

that species to be filtered out include non-desirable polypeptides, proteins, cellular components, DNA, colloids, mycoplasm, endotoxins, viruses, carbohydrates, and other molecules of biological interest, whether glycosylated or not.

5 **Tangential Flow Filtration**

A process in which the fluid mixture containing the components to be separated by filtration is re-circulated at high velocities tangential to the plane of the membrane to increase the mass-transfer coefficient for back diffusion. In such filtrations a pressure differential is applied along the length of the membrane to cause the fluid and filterable solutes to flow through the filter. This filtration is suitably conducted as a batch process as well as a continuous-flow process. For example, the solution may be passed repeatedly over the membrane while that fluid which passes through the filter is continually drawn off into a separate unit or the solution is passed once over the membrane and the fluid passing through the filter is continually processed downstream.

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Transmembrane Pressure

The pressure differential gradient that is applied along the length of a filtration membrane to cause fluid and filterable solutes to flow through the filter. In tangential flow systems, highest TMP's are at the inlet (beginning of flow channel) and lowest at the outlet (end of the flow channel). TMP is calculated as an average pressure of the inlet, outlet, and filtrate ports.

Recovery

25 The amount of a molecule of interest that can be retrieved after processing. Usually expressed as a percentage of starting material or yield.

Retentate

The portion of the sample that does not pass through the membrane, also known as the concentrate. Retentate is being re-circulated during the TFF.

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Principles of Tangential Flow Filtration

[0040] There are two important variables involved in all tangential flow devices: the transmembrane pressure (TMP) and the crossflow velocity (CF). The 35 transmembrane pressure (TMP) is the force that actually pushes molecules through the pores of the filter. The crossflow velocity is the flow rate of the solution across the membrane. It provides the force that sweeps away larger molecules that can clog the membrane thereby reducing the effectiveness of the process. In practice a fluid feedstream is pumped from the sample feed container source across the membrane 40 surface (crossflow) in the filter and back into the sample feed container as the retentate. Backpressure applied to the retentate tube by a clamp creates a transmembrane pressure which drives molecules smaller than the membrane pores through the filter and into the filtrate (or permeate) fraction. The crossflow sweeps larger molecules, which are retained on the surface of the membrane, back to the feed as retentate. The primary

Step # 1 (Clarification)

- [0042] Turning to FIG. 1, transgenic mammal milk, preferably of caprine or bovine origin, is clarified utilizing batch-wise microfiltration. The milk is placed into a feed tank and pumped in a loop to concentrate the milk retentate two fold (see flow diagram in FIG. 1). Once concentrated the milk retentate is then diafiltered allowing the product and small molecular weight proteins, sugars, and minerals to pass through an appropriately sized membrane. According to the current invention, this operation is currently designed to take 2 to 3 hours and is will process 1000 liters of milk per day.
- 5 The techniques and methods of the current invention can be scaled up and the overall volume of product that can be produced is dependent upon the commercial and/or therapeutic needs for a specific molecule of interest.
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15 Step # 2 (Concentration / Fractionation)

- [0043] Again referring to FIG. 1., the clarified permeate from the first step is concentrated and fractionated using ultrafiltration (“UF”). The clarified permeate flows into the UF feed tank and is pumped in a loop to concentrated the product two-fold.
- 20 Once the concentration step is initiated the permeate from the UF is placed into the milk retentate in the clarification feed tank in the first step. The first and second step are sized and timed to be processed simultaneously. The permeate from the UF contains small molecular weight proteins, sugars, and minerals that pass through the membrane. Once 95% of the product is accumulated in the retentate of the UF, the
- 25 clarification is stopped and a concentration / diafiltration of the UF material is begun. The product is concentrated 5 to 10 fold the initial milk volume and buffer is added to the UF feed tank. This washes away the majority of the small molecular weight proteins, sugars, and minerals. This operation is currently designed to take 2.5 to 3.5 hours and can process upto 500 liters of clarified permeate per day. As above, the
- 30 techniques and methods of the current invention can be scaled up and the overall volume of product that can be produced is dependent via this concentration/fractionation process is dependent upon the commercial and/or therapeutic needs for a specific molecule of interest.

Step # 3 (Aseptic filtration)

[0044] According to FIG. 1., and according to the current invention, the clarified bulk concentrate is then aseptically microfiltered. The resulting 50 to 100
5 liters of UF retentate is placed into a feed tank where it is pumped through a dead-end absolute 0.2 µm MF filtering system in order to remove the majority of the bioburden and enhance stability of the product for extended periods of time. The product is pumped through the filtering system of the invention and may then be directly filled into a final packaging configuration. Under conditions for processing a molecule of
10 interest in a GMP facilities meeting clean room specifications (e.g., class 100 conditions) This operation is currently designed to take 0.5 to 1 hour and will process upto 100 liters of clarified bulk intermediate per day. As above, the techniques and methods of the current invention can be scaled up and the overall volume of product that can be produced is dependent via this concentration/fractionation process is
15 dependent upon the commercial and/or therapeutic needs for a specific molecule of interest.

EXAMPLE 1**MILK AS A FEEDSTREAM FOR THE PRODUCTION OF A MOLECULE OF INTEREST**

[0045] The data below provides an application of the current invention that provides a membrane-based process to clarify, concentrate, and fractionate transgenically produced an IgG1 antibody from a raw milk feedstream. According to
25 this example of the invention the transgenic mammal providing the milk for processing was a goat but other mammals may also be used including cattle, rabbits, mice as well sheep and pigs. Initial operational parameter ranges for processing were optimized utilizing CHO-cell produced IgG1 antibodies spiked into non-transgenic goat milk. When a transgenic goat capable of producing this molecule of interest came into
30 lactation and began producing recombinant IgG1 antibodies in its milk, the several experiments were performed using CHO-cell produced recombinant IgG1 antibodies spiked into non-transgenic milk and were repeated with transgenic milk.

[0046] Pursuant to the current invention the experimental strategy was to determine the relationships between the filtration process variables that can be controlled on a large scale, (CM, V, TMP, T), where V is Flow Velocity, as can product
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Sample #1

[0082] This sample was taken from milk spiked milk. The permeate line of the MF is fed to the feed reservoir. At time equals 10 min the permeate is directed through path "B" (permeate to drain). This will concentrate the milk to 500 ml. Once the milk is 2x the original concentration, the permeate is switched back to path "A" (re-circulation back to feed reservoir). After 10 min in re-circulation, sample numbers 2 and 3 are taken then the back pressure valve is adjusted to cause the feed pressure near the pump to read 10 psi. Feed flow rate is maintained at 13.35 l/min by adjusting the pump speed to 55 Hz. After 10 min in re-circulation, sample numbers 4 and 5 are taken and the back pressure valve is adjusted to cause the feed pressure near the pump to read 14 psi. Feed flow rate is maintained at 13.35 L/min by adjusting the pump speed to 60.66 Hz. After 10 min in re-circulation sample numbers 6 and 7 are taken and the back pressure valve is adjusted to cause the feed pressure near the pump to read 12 psi. Feed flow rate is adjusted to 7.75 L/min by adjusting the pump speed to 40 Hz. After 10 min in re-circulation sample numbers 8 and 9 are taken then the back pressure valve is adjusted to cause the feed pressure near the pump to read 14 psi.

[0083] Feed flow rate is maintained at 7.75 l/min by adjusting the pump speed to 43.45 Hz. After 10 min in re-circulation, sample numbers 10 and 11 are taken and the back pressure valve is adjusted to cause the feed pressure near the pump to read 10 psi. Feed flow rate is adjusted to 12.36 L/min by adjusting the pump speed to 48 Hz. After 10 min in re-circulation sample numbers 12 and 13 are taken and the back pressure valve is adjusted to cause the feed pressure near the pump to read 14 psi. Feed flow rate is maintained at 12.36 L/min by adjusting the pump speed to 55.44 Hz. After 10 min in re-circulation sample numbers 14 and 15 are taken then the back pressure valve is adjusted to cause the feed pressure near the pump to read 20 psi. Feed flow rate is adjusted to 12.36 l/min by adjusting the pump speed to 61.69 Hz. After 10 min in re-circulation, sample numbers 16 and 17 are taken and the feed flow rate is adjusted to read 13.35 L/min, 64.64Hz, and the back pressure valve is adjusted to cause the feed pressure near the pump to read 20 psi. After 10 min in recirculation, sample numbers 18 and 19 are taken and the feed flow rate is adjusted to 7.75 L/min by adjusting the pump speed to 52.65 Hz and the back pressure valve is adjusted to cause the feed pressure near the pump to read 20 psi. After 10 min in re-circulation sample numbers 20 and 21 are taken and the pump is turned off, and the pump is turned off. All

- [0088] Though spiking CHO-cell IgG1 antibody into non-transgenic milk gave an initial look at the behavior of IgG1 antibody in milk, naturally lactated milk containing the IgG1 antibody required different optimization parameters for the use of a 5 0.2 mm ceramic membranes preferably used according to the current invention. The spiking study showed a lower optimum flow velocity and very high product fluxes than the transgenic milk study. Moreover, running the dual TFF system using the parameters optimized for transgenic milk provided lower product recovery for natural non-transgenic milk gave spiked with the IgG1 antibody.
- 10 [0089] Tangential flow filtration (TFF) was implemented as a process to clarify and stabilize IgG1 antibody in a milk matrix by removing particulate matter such as fat, casein micelles, and bacteria from raw milk. TFF is widely used in both the biotechnology and dairy industries to remove impurities and concentrate product. In order to use TFF effectively according to the current invention it is important that the 15 proper membranes are chosen, the process parameters (temperature, trans-membrane pressure, cross-flow velocity, and milk concentration) are optimized for high product flux, and the cleaning and storage procedures were developed to ensure long membrane life. Experimental matrix parameters are described herein, according to the current invention and applied to transgenic goat milk to confirm previous operational parameters. Membrane cleaning and storage conditions were also investigated. An 20 aseptic filtration step was developed to remove any bacteria remaining from the clarified milk product containing a protein of interest after the TFF process is complete. Process information was then transferred to pilot scale equipment were initial engineering runs were conducted. Some process design criteria included, using no 25 additives to prevent the need for water for injection, long membrane life, high yield, and short processing time. The process of the current invention was preferably designed to be scalable for pilot and manufacturing operations.

30 **Process Description**

[0090] To perform dual TFF using a ceramic 0.2 μm microfiltration membrane and a 30 kDa ultrafiltration membrane to clarify and concentrate transgenic goat milk from goat D035, the system was sanitized with 0.1M sodium hydroxide. Then the milk must be pooled and raised to 15-20 °C. The milk must be concentrated

Table 1.**Ceramic membrane cleaning steps:**

		<u>Step</u>	<u>Concentration</u>	<u>Volume</u>	<u>Time</u>	<u>Temp</u>	<u>pH</u>
5	1)	Water Flush	-	16-20L	5 min.	60 °C	7.0
	2)	NaOH Wash	0.5 M	1	10 min.	60	>11.5
	4)	Sodium Hypochlorite	400 ppm				
	4)	NaOH Wash	0.5 M	1	30 min.	60	>11.5
		Sodium Hypochlorite	400 ppm				
10	5)	Water Flush	-	20-25	5 min.	60	7.0
	6)	Citric Acid Wash	0.4 M	1	30 min.	60	<2.75
	7)	Water Flush	-	16	10 min.	60	7.0
	8)	Sodium Hypochlorite	300 ppm	1	15 min.	60	>9.5
		NaOH	0.05 M				
15	9)	Water Flush	-	12	10 min.	60	7.0
	10)	NaOH Storage	0.1 M	1		20	10-12

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Table 2.**30kDa PES membrane cleaning steps:**

		<u>Step</u>	<u>Concentration</u>	<u>Volume</u>	<u>Time</u>	<u>Temp.</u>	<u>pH</u>
25	1)	USP Water Flush	-	2L/sqft		35 °C	5.0
	2)	NaOH Flush	0.5 M	2L/sft		35	>11.5
	4)	Sodium Hypochlorite	250ppm				
	4)	NaOH Wash	0.5 M	2L/sqft	60 min.	35	>11.5
		Sodium Hypochlorite	250 ppm				
30	5)	USP Water Flush	-	4L/sqft		35	7.0
	6)	Citric Acid Wash	0.4 M	2L/sqft	60min	35	<2.75
	7)	USP Water Flush	-	4L/sqft		35	7.0
	10)	NaOH Storage	0.1 M			35	10-12

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[0099] Prior to using a membrane for the first time, a normalized water permeability curve was made relating transmembrane pressure, temperature and water flux. Prior to use in an experiment, the normalized water permeability was checked to maintain a minimum 80% recovery of water flux. The ceramic membranes maintained a 95-105% recovery during development and the 30kDa PES membranes maintained 80-90% recovery.

45 Aseptic Filtration

[00100] As seen in Graph J below, Pall Gelman Inc., makes a sterile filter made of Supor membrane with 0.8um prefilter membranes and 0.2um filter membranes

and permeate lines with a cross flow rate of 1LPM, then the permeate is flushed with an additional 3L.

- [00117] Using USP water (adding more if necessary), pump the MF at 20LPM, increase the retentate pressure until the TMP of 15psi is reached with no permeate
- 5 pressure, then adjust the cross flow rate with pump speed to 15LPM. Record the temperature (must be between 25-28 °C), pressures, and cross flow rate. Measure the permeate flow rate through the permeate drain valve. Repeat on the UF using 1 LPM of cross flow, and 5 psig of retentate pressure, and no permeate pressure (TMP of approximately 10psig). Compare the permeate flow rates to those of the membranes' 10 virgin water permeability. If the permeation rate is less than 80% of the original value, either re-clean the membranes or replace them.

Milk Processing

- 15 [00118] The milk must be pooled and raised to 15-20 C. The milk is pooled in the MF reservoir, then the MF permeate valve is closed, the retentate valve is opened, and the pump is turned on for a cross flow of 20LPM. After 5 minutes the initial milk sample(s) are taken. The pressure is then increased for a TMP of 15 psig and cross flow rate of 15 LPM. The recirculation continues until the milk temperature reaches 20 °C. Then the chiller is turned on at 10 °C and the MF permeate valve is opened to allow the milk to be concentrated to half of it's original volume on the microfiltration system by collecting the permeate of the ceramic membrane. The MF is run at 15 lpm cross flow rate with 15psi of transmembrane pressure. The temperature of the MF should increase to and remain at 26 °C ± 2.0. The ultrafiltration system must then be 20 started up at 0.8-1 LPM/sqft cross flow rate. The permeate flow rates of each membrane are measured through the permeate valves. The retentate and permeate pressures of the UF must be adjusted to cause the permeate flow rate to match the permeate flow rate of the MF. Once the UF permeate flow rate matches that of the MF. The systems should be run coupled for 5-6 diafiltration volumes. Once diafiltration is 25 complete, the systems are disconnected, the MF is shut off, drained and cleaned, and the UF permeate is directed to drain until the volume of bulk clarified concentrate in the feed reservoir of the UF is concentrated to half it's volume for a total concentration of 4

X. The UF is then drained, the bulk clarified concentrate is aseptically filtered, and the UF is cleaned.

5 Cleaning and Storing Protocols

[00119] The systems are disconnected according to the diagrams on page 14 of this report. The MF is rinsed with 20 L hot soft water (45-65 °C) with the retentate valves half open, and the permeate directed to drain. The valves are directed to recirculate solution back to the feed reservoir, and 2 L of hot 0.5 M sodium hydroxide with 400 ppm sodium hypochlorite is re-circulated for 5 minutes. The solution is drained from the system and replaced with 2 L of the same chemicals. The fresh solution is re-circulated for 30 minutes, then drained through the bleed valve. The system is flushed with 20 L of hot soft water through the half opened retentate valves. 4 L is flushed through the permeate only by recirculating the water on the retentate side of the membrane at 20 lpm with 6-8 psi of TMP. Remaining water is drained through the bleed valve. 2 L of hot 0.5 M citric acid is re-circulated through the system for 30 min at 20 LPM with 6-8 psi of TMP. The citric acid is then drained out through the bleed valve. 15 L of soft water is used to rinse out the retentate side of the MF, and 4 L is used to rinse out the permeate side as was done after the caustic step. 2 L of hot 0.05 M sodium hydroxide with 400 pm bleach was then re-circulated through the MF for 15 minutes and drained and rinsed out with 10 L of water on the retentate side and 4 L through the permeate as was done after the caustic step. The UF retentate and permeate lines are directed to drain for the initial water flush by directing the retentate valve to drain, and directing the entire permeate line to drain (not by the valve). Always run the pump at 1LPM, i.e. if the retentate pressure is increased, the pump speed must also be increased to maintain 1LPM. Rinse 4 L of USP water through both lines. Flush 2 L of 0.5 M sodium hydroxide with 250 ppm sodium hypochlorite made with USP water through both lines. Recirculate 2 L of fresh solution through the system with the permeate line attached to the feed reservoir, and the retentate valve open to the reservoir for 60 minutes. Drain the solution through the bleed valve. Direct both lines to drain as in the initial flush. Fill the reservoir with USP water and drain 1 L through the bleed valve. Flush 8 L through both lines, and an additional 4 L through the permeate line with 5 psi of retentate pressure. 2 L of 0.4 M citric acid are then re-circulated through the system for 60 minutes. The acid solution is drained through the